Dietary (n-3) Fatty Acids from Menhaden Fish Oil Alter Plasma Fatty Acids and Leukotriene B Synthesis in Healthy Horses

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Abstract:

The study objective was to determine the effect of feeding corn oil or fish oil to horses on plasma fatty acid profiles and leukotriene B (LTB) synthesis by stimulated peripheral blood neutrophils. Two groups of horses (n = 5) were randomly assigned to diets supplemented with either 3.0% (by weight) corn oil or fish oil for a period of 14 weeks. The ratio of (n-6) to (n-3) fatty acids in oil supplements was 68.1 : 1 for corn oil and 0.12: 1 for fish oil. Production of LIB, and LIB, by peripheral blood neutrophils stimulated with calcium ionophore A23187 and plasma cholesterol, triacylglycerol, and α-tocopherol concentrations were measured. oil supplements was 68.1 : 1 for corn oil and 0.12: 1 for fish oil. Production of LIB, and LIB, by peripheral blood neutrophils stimulated with calcium ionophore A23187 and plasma cholesterol, triacylglycerol, and α-tocopherol concentrations were measured. At 12 weeks, horses fed fish oil had increased plasma concentrations of eicosapentaenoic acid (27-fold; 8.5 versus 0.3 g/100 g fatty acids; P < .0001), docosahexaenoic acid (34-fold; 5.1 versus 0.1 g/100 g fatty acids; P < .0001), and arachidonic acid (8.3- fold; 4.1 versus 0.5 g/100 g fatty acids; P < .0001) compared with horses fed corn oil. Neutrophils from horses fed fish oil produced 78-fold (P = .01) more LTB5 and 9.5-fold (P = .003) more LTB, compared with predietary levels, and 17.6-fold (P = .01) and 3.3-fold (P = .02), respectively, more than horses fed corn oil, and the ratio of LIB, to LIB, concentrations was 4.0-fold (P = .002) higher in horses fed fish oil. This study suggests that dietary polyunsaturated fatty acids modulate the leukotriene inflammatory response of horses. If the ratio of LTB5 to LIB, concentrations is important in determining how inflammatory processes are mediated, then fish oil supplementation may have value in treatment of equine inflammatory diseases.

Article:

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Linoleic acid and α-linolenic acid, both essential fatty acid and α-linolenic acid, both essential fatty acids, are the respective precursors of the (n-6) and (n-3) series of fatty acids. Mammals lack enzymes to introduce double bonds at carbon atoms before the ninth carbon atom in the fatty acid chain, counting from the methyl end, and therefore, these fatty acids must be supplied in the diet.' z Linoleic acid, an (n-6) polyunsaturated fatty acid (PUFA), is found in corn, safflower, and soybean oils, whereas α-linolenic acid, an (n-3) PUFA, is found in linwhereas α-linolenic acid, an (n-3) PUFA, is found in linseed, canola, and soybean oils. Further metabolism of linoleic acid (eg, elongation and desaturation) produces arachidonic acid (AA), which is subsequently incorporated into cell membrane phospholipids. Further metabolism of α-linolenic acid produces eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are also incorporated into cell membrane phospholipids. In fish oil, the predominant (n-3) fatty acids are EPA and DHA. It is believed that by increasing the amount of dietary (n-3) PUFA relative to (n-6) PUFA, that (n-3) fatty acids are preferentially incorporated into cell membrane phospholipids compared with (n-6) fatty acids.[1-6]

When cells are activated by a chemical or physical insult, fatty acids are mobilized from cell membrane phospholipids and metabolized into eicosanoids, which mediate inflammatory processes. Further metabolism of AA by cyclooxygenase leads to production of proinflammatory eicosanoids of the 2-series (eg, thromboxane A_2 and prostaglandin [PG] E_2), whereas further metabolism of AA by lipoxygenase yields the 4-series eicosanoids (eg, leukotriene [LT] B_4).[2-7] PGE_2 has a number of proinflammatory effects, including induction of fever and erythema, increased vascular permeability and vasodilation, and enhancement of pain and edema caused by other agents such as bradykinin and histamine. PGE_2 also regulates the production of cytokines by immune cells. Leukotrienes have chemotactic properties and are involved in regulation of inflammatory and immune processes. Leukotrienes of the 4 series regulate cytokine production.

Alternatively, further metabolism of EPA produces less inflammatory eicosanoids of the 3 and 5 series (eg, thromboxane A_3, PG_3, and LTB_5).[2-7] The eicosanoids produced from EPA are often less biologically potent than analogues synthesized from AA. For example, LTB_5 is 10-fold less potent as a neutrophil chemoattractant than LTB_4.[3-7] Thus, the type of eicosanoids that cells produce, and consequently, communication between cells of the immune system, can potentially be modulated through dietary supplementation of essential fatty acids.[3-7]

The goals of this investigation were (1) to determine whether plasma fatty acid profiles, serum lipids, or serum biochemistries are altered by dietary fatty acid supplementation in horses similar to that observed in other species; and (2) to determine the effect of feeding different amounts of (n-3) fatty acids on leukotriene production by stimulated peripheral blood neutrophils from horses. We wanted to determine whether dietary supplementation with fish oil reduced expression of proinflammatory leukotrienes in healthy horses. To achieve this goal, we measured LTB_4 and LTB_5 production in calcium-ionophore-stimulated peripheral blood neutrophils of horses fed either corn oil or fish oil.

Table 1. Composition of selected fatty acids of the oils used in the two horse experimental diets.

Oil Supplements (n-6):(n-3)	Corn Oil 68.1:1	Fish Oil 0.12:1
Fatty Acid	g/kg of Oil	
C14:0	ND[a]	58.9
C16:0	101.2	150.9
C18:0	18.6	27.0
Sum SFA[b]	119.8	241.3
C16:1(n-7)	ND	85.1
C18:1(n-9)	253.9	107.3
Sum MUFA[c]	253.9	225.5
C18:2(n-6)	565.5	12.1
C18:3(n-3)	8.3	10.5
C18:4(n-3)	ND	2.3
C20:4(n-6)	ND	6.7
C20:5(n-3)	ND	84.3
C22:5(n-3)	ND	13.3
C22:6(n-3)	ND	72.0
Sum PUFA[d]	573.8	203.8
Sum (n-6)[e]	565.5	21.4
Sum (n-3)[f]	8.3	182.4

[a] ND, not detectable.

[b] Sum of the saturated fatty acids: 8:0 + 10:0 + 11:0 + 12:0 + 14:0 + 15:0 + 16:0 + 18:0 + 20:0 + 22:0 + 24:0.

[c] Sum of the monounsaturated fatty acids: 14:1 + 15:1 + 16:1(n-7) + 17:1 + 18:1(n-9) + 18:1(n-7) + 20:1(n-9) + 22:1(n-9) + 24:1.

[d] Sum of the polyunsaturated fatty acids: 18:2(n-6) + 18:3(n-6) + 18:(n-3) + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:4(n-3) + 20:5(n-3) + 21:5(n-3) + 22:2(n-6) + 22:4(n-6) + 22:5(n-6) + 22:5(n-3) + 22:6(n-3).

[e] Sum of the (n-6) fatty acids.

[f] Sum of the (n-3) fatty acids.

MATERIALS AND METHODS

Animals

Ten mature light-breed mares ranging in age from 5 to 20 years (those fed corn oil, 12.4 ± 0.7 years; those fed fish oil, 13.8 ± 2.9 years; mean ± SEM) and weighing between 429 and 553 kg (mean 502 kg) were selected for this study from the teaching herd at the College of Veterinary Medicine, Oregon State University, Corvallis, OR. All mares were nonpregnant and nonlactating throughout the duration of the study. Horses were determined to be healthy on the basis of physical examinations, complete blood counts, and serum biochemical evaluations completed at study initiation and throughout the study duration. All horses had been dewormed every other month and vaccinated according to current recommendations for encephalomyelitis, tetanus, influenza, and rhinopneumonitis. A commercially available oral paste dewormera was used to control internal parasites.

Diets

Experimental diets were designed to be isocaloric and isonitrogenous with all other minerals and vitamins equal between groups and balanced to meet or exceed current National Research Council recommendations for maintenance.[8] The two experimental diets differed only in the oil source used to manipulate the proportion of (n-3) and (n-6) fatty acids. Diets contained either feed-grade Menhaden fish oil[a] for the (n-3) enriched diet, or corn oil[c] for the (n-6) enriched diet (Table 1). Oils were refrigerated and containers were capped with nitrogen. Peroxide values and

p-anisidine values, a measure of the al dehyde content of fat, were measured to assess the degree of oil per- oxidation.[9]

Dietary components (on an as-fed basis) included grass hay (86.0%), beet pulp (10.1%), either corn oil or fish oil (3.0%), vitamin- mineral supplement' (0.6%), and limestone (0.3%). White salt was freely available to all mares. Grass hay, beet pulp, and oils were obtained from single sources in sufficient quantity to last for the study duration. Calculated nutrient composition (dry matter basis) of experimental diets based on individual feed analyses[e] was 8.5% crude protein, 2.2 Mcal/kg digestible energy, 4.9% ether extract, 56.0% neutral detergent fiber, 35.2% acid detergent fiber, 0.34% calcium, and 0.25% phosphorus. Mares were expected to consume between 7.7 and 9.5 kg of dry matter per day depending on body weight. Beet pulp, oil, and mineral sources were fed at a defined set amount, whereas hay intake was allowed to fluctuate to meet daily needs. With the allowed variation in hay intake, based on body weight differences, fat sources contributed between 12 and 15% of digestible energy calories (2.45 Mcal/d). Both grass hay (2.0% ether extract) and beet pulp (0.6% ether extract) feeds contained <2.0% total fatty acids; therefore, supplemental oil sources accounted for >98% of dietary fatty acids consumed.

Horses were acclimated to a diet containing beet pulp without oil for 2 weeks before the study initiation. During the dietary intervention period, mares were allowed access to an exercise area and provided hay ad libitum during the day and housed in stalls in the evening when study diet supplements were fed. Beet pulp (0.91 kg as fed) was soaked in water for 6 to 8 hours before being mixed with either corn oil or fish oil (272 g; approximately 300 mL). Total (n-3) fatty acids comprised 0.88% of total dietary fatty acids (2.3 g/d) for horses fed the corn oil supplement and 27.2% (49.6 g/d) for horses fed the fish oil supplement. Oils were warmed to room temperature before feeding. Vitamin-mineral supplement (56 g) and limestone (15 g) were also added to the beet pulp and oil mixture. Horses were fed the supplement mixture in the afternoon, feed buckets remained in the stalls overnight, and food consumption was assessed the following morning.

Study Design

Horses were randomly assigned to 1 of 2 feeding groups (n = 5). Horses were fed oil-supplemented diets for a period of 14 weeks following the diet acclimation period. Oil supplements were discontinued at 14 weeks. The time from week 14 to week 18 served as a washout period. Dietary supplement consumption was recorded daily throughout the feeding period. Body weights were recorded once prestudy, weeldy during the first 3 weeks of the feeding trial, and then biweeldy through week 12. Jugular venous blood was collected into tubes with and without ethylenediamine tetraacetic acid at 0, 6, 8, 12, and 18 weeks. Plasma was stored at —70°C until plasma fatty acid profiles and cholesterol, triacylglycerol, and α-tocopherol concentrations were and cholesterol, triacylglycerol, and α-tocopherol concentrations were determined. The experimental protocol was reviewed and approved by the Oregon State University Animal Care and Use Committee according to principles outlined by the National Institutes of Health.[10]

In addition to collection of peripheral blood (for studies reported in this paper), we also investigated selected immune responses in vivo, and selected immune responses of cells isolated from airways of these horses. These results are reported in a companion paper.[11] Horses were

challenged with a foreign protein (keyhole limpet hemocyanin) at 8 and 10 weeks after initiation of the feeding trial. A delayed-type hypersensitivity skin test was performed during week 10. All horses were treated similarly.

Leukotriene B4 and Bs Quantification

Neutrophils were isolated and purified as previously described from blood collected at 0 and 12 weeks of the study.12 Aliquots of 1 X 10' neutrophils were transferred to 5-mL polypropylene tubes and the volume was adjusted to 495 with Hank's balanced salt solution containing 0.8 mmol/L CaCI,, Neutrophils were then stimulated with 5 of calcium ionophore A23187' in 0.2% dimethyl sulfoxide' such that the final concentration of A23187 was 10 µmol/L, while unstithat the final concentration of A23187 was 10 µmol/L, while unstimutated PMN received 5 of 0.2% dimethyl sulfoxide without calcium ionophore. All tubes were incubated for 5 minutes in a 37°C water bath and the reaction was terminated by addition of 2 mL of ice-cold methanol to each tube and incubation on ice for 20 minutes. Tubes were centrifuged for 5 minutes at 1000 X g, and supernatants were transferred to 5-mL polypropylene tubes and stored at —70°C until subsequent LIB_4 and LTB_5 measurements were made.

Leukotrienes were extracted and separated using a modified version of methods described by Terano et al[13] and Vaughn et al.[14] Stored supernatants from the leukotriene stimulation procedure were centrifuged for 5 minutes at 400 X g and transferred to 15-mL centrifuge tubes. Prostaglandin B_3[h] (100 ng), which served as an internal standard, was added to each sample before extraction. Citrate buffer (pH 4.0) was added to achieve a final volume of 14.5 mL. Samples were loaded onto an activated C-18 solid-phase extraction cartridge by gravity flow. Cartridges were rinsed with 5 mL of distilled-deionized (dd)-H20 followed by 5 mL of high-performance liquid chromatography (HPLC)-grade hexane. Leukotrienes were eluted using 5 mL of a methanol: dd-$H_2$0 mixture (90v : 10v) by gravity flow into 5-mL polypropylene tubes. Tubes were placed in a 30°C water bath and the eluate was evaporated under a stream of N_2. Residues were reconstituted in 125 µl of mobile phase (methanol: water : glacial acetic acid [75 : 25 : 01], pH adjusted to 5.7 with NH_4,OH), capped with N, and stored at —70°C until separation by HPLC.

Leukotrienes B_4 and B_5 were separated by HPLC using a C-18 reversed-phase column[j] fitted with a C-18 guard column.[k] The mobile phase was methanol: water : glacial acetic acid (75 : 25 : 01), and pH was adjusted to 5.7 with NH_2OH. Flow rate of the pump[l] was set at 0.7 mL/min and the variable wavelength UV detector[m] was fixed at 254 nm.

Elution times were determined using PGB_3, LTB5, and LIB_4, standards,[h] and sample fractions were collected from 6.2 to 8.0 minutes for LTB, and from 8.5 to 10.3 minutes for LTB_5. Fractions were also collected at the start (0 to 6.2 minutes) and end (10.3 to 15 minutes) of each run and between leukotriene fractions (8.0 to 8.5 minutes). The latter were analyzed to ascertain that they did not contain leukotriene. Fractionated samples were capped with N_2 and stored at —70°C for subsequent analysis by enzyme immunoassay (EIA). Peaks were integrated using an integrator.[n]

Concentrations of LTB_4 and LTB_5 in samples were determined using EIA kits[h] Samples obtained from the HPLC separation of leukotrienes were placed in a 37°C water bath and evaporated to

dryness under a stream of N_2. Samples were then reconstituted in 150 of EIA buffer and stored at 4°C until they were assayed by EIA according to the manufacturer's instructions. All samples were analyzed in duplicate and plates were read when a maximum binding well reached an optical density (O.D.) of 0.500. The O.D. of each well at 405 nm was determined using a Bio-Tek EL312 microplate reader.[o] The antiserum that was used had a cross-reactivity of 100% for LIB_4 and 50% for LTB_5. An additional standard curve for LTB_5, was prepared in the same manner as the LTB_4, standard to accurately quantify LTB_5. Concentrations for LTB_4 and LTB_5 were calculated using the software program Kinetic Cale version 2:12.[o]

Other Chemical Assays

Fatty acid profiles were determined by gas chromatography as previously described[15] using heptadecanoic acid as the internal standard. Fatty acid concentration was expressed as g/100 g fatty acids. Plasma α-tocopherol content was measured by HPLC using a fluorometric detector and expressed as μmol/L and μmol/mmol lipid.[9] Plasma concentrations of cholesterol and triacylglycerol were determined enzymatically by methods previously discussed.[9] Total plasma lipid content was described as the sum of cholesterol and triacylglycerol concentrations.[16] Serum biochemistries were analyzed at 0, 8, and 12 weeks using a Roche FARA II system.[p] Measured serum analytes included urea nitrogen, creatinine, glucose, total protein, albumin, total bilirubin, creative kinase, gamma-glutamyltranspeptidase, aspartate aminotransferase, sodium, potassium, chloride, calcium, phosphorus, magnesium, and sorbitol dehydrogenase.

Statistical Analysis

The trial was a completely randomized design. Using the Kolmogorov-Smirnov and Cramer-von Mises tests, all data were found to be normally distributed. Data were analyzed using the MIXED procedure of Statistical Analysis Systems for repeated measures." Main effects were diet, week, and their interaction analyzed over time. Because of differences in initial body weight among horses, initial body weight was used as a covariate in all models, but was dropped from final models because it was found to be not significant. For each dependent variable, a horse nested in treatment (diet) was subjected to differing covariance structures. The best model fit was determined by lowest parameter values for covariance structure. For main effects found to be significant, mean differences were determined by pairwise differences or probability values for differences of the least-squares means (PDIFF) for preplanned comparisons.[17] Data are reported as least square means ± SEM unless otherwise indicated. A two-sample t-test was used to compare leukotriene data. Values were considered significant at P .05 unless otherwise indicated.

RESULTS

Animals and Diets

All horses readily consumed the oil-enriched diets, and weekly food consumption (measured as a percentage of amount fed) was calculated to be greater than 95% for both groups of horses throughout the study. One horse in the fish oil group had decreased food consumption (25 to 50%) beginning the 10th week of the feeding trial, which lasted through the end of the feeding period. At week 12, complete blood count (CBC) results from this horse exhibited neutrophilia with left shift. Given the implication of an active inflammatory process, data from this horse for weeks 8 and 12 were excluded. For all other horses, the results of serial CBCs (data not shown) and serum biochemistry measures were within established reference intervals for normal animals and, thus, were interpreted to suggest that all animals were healthy and free of obvious

inflammatory disease. Significant oxidation of oils at the beginning or end of the feeding trial was not detected based on peroxide and p-anisidine values.

Initial body weight was used as a covariate in all statistical models. Initial mean body weight for horses fed fish oil fed was 542 ± 13 kg, and for horses fed corn oil it was 477 ± 13 kg. All horses gained body weight during the first 4 weeks of the study, then lost weight, such that body weights from week 7 through the end of the study were not different from initial body weights. Thus, net change in body weight across time was not influenced by diet, nor was average daily body weight change.

Plasma Fatty Acid Profiles

There were no major differences in plasma (n-6) and (n3) fatty acid compositions between horses at the beginning of the study. Plasma fatty acid profiles showed significant changes 6 weeks after supplementation began with corn oil or fish oil (Table 2). In general, changes noted in the plasma fatty acid profiles at 6 weeks persisted through 12 weeks. Horses that consumed the corn oil supplement had a 29% increase in plasma (n-6) fatty acids at 6 weeks that persisted at 8 and 12 weeks, and no change in plasma (n-3) fatty acids. Conversely, horses consuming the fish oil supplement showed a 24% decrease in plasma (n-6) fatty acids at 6 weeks, with marked increases in plasma concentrations of total (n-3) fatty acids at 6, 8, and 12 weeks compared with baseline. Thus, horses fed fish oil had higher concentrations of (n-3) fatty acids than horses fed corn oil at 6, 8, and 12 weeks.

The calculated ratio between (n-6) and (n-3) fatty acids in plasma was not different between treatment groups at baseline. In corn oil—fed horses, the (n-6) to (n-3) fatty acid ratio was increased at 6, 8, and 12 (highest) weeks of the study compared with week 0. In contrast, fish oil—fed horses showed a marked decline in (n-6) to (n-3) fatty acid ratio from week 0 to 6 and 8 weeks. At 12 weeks, it was not different from baseline.

Before beginning corn oil or fish oil supplementation, linoleic acid made up the largest percentage of the total plasma PUFA in both groups of horses. After feeding horses the corn oil supplement, there was an increase in plasma linoleic acid at 6 weeks from the baseline value. This change persisted through 8 and 12 weeks, but returned to pretrial levels 4 weeks after discontinuing supplementation. However, in horses fed fish oil, there was a decrease in plasma linoleic acid at 6, 8, and 12 weeks, which persisted even after discontinuing the oil supplements (18 weeks).

Fish oil—fed horses showed increased plasma AA, EPA, and DHA concentrations compared with corn oil—fed horses. Plasma levels of AA, EPA, and DHA were decreasing by 18 weeks (4 weeks after discontinuing the fish oil supplement), although AA and DHA concentrations were still higher than baseline. Corn oil—fed horses showed no change in plasma concentrations of AA, EPA, or DHA over time. Plasma concentrations of α-linolenic acid were decreased Plasma concentrations of α-linolenic acid were decreased in horses from both groups at 6, 8, and 12 weeks compared with baseline values, but returned to baseline values at 18 weeks.

Plasma concentrations of total saturated fatty acids (SFA) were decreased at 6 and 8 weeks from baseline values in the corn oil—supplemented horses. No significant differences in plasma total

SFA were noted between the 2 groups of horses. The SFA 16 : 0 and 22 : 0 were significantly different between the 2 groups of horses at 6 and 8 weeks (also at 12 weeks for 22 : 0), with horses fed fish oil supplements having higher concentrations of each.

In general, horses fed fish oil supplements had higher concentrations of monounsaturated fatty acids (MUFAs) compared with horses fed corn oil supplements, although total MUFAs decreased in horses from both diet groups and remained lower than baseline levels at 6, 8, and 12 weeks. Four weeks after discontinuing the fatty acid supplements (18 weeks), plasma concentrations of MUFAs increased in both groups of horses, but remained decreased from baseline in the corn oil—fed horses.

Plasma Cholesterol, Triacylglycerol, α-Tocopherol

Mean plasma cholesterol concentrations were not different between horses fed corn oil and horses fed fish oil at any time point. Across both diets, plasma concentrations were lowest at week 0, greatest at week 6, and weeks 8 and 12 were equivalent with both means being lower than week 6 (Table 3). Mean plasma triacylglycerol concentrations were not influenced by dietary treatment. Plasma triacylglycerol was highest at week 0 compared with weeks 6 and 12, but not different from week 8. Plasma α-tocopherol 12, but not different from week 8. Plasma α-tocopherol concentration increased at week 6 compared with week 0 in both groups of horses and remained elevated at weeks 8 and 12. When plasma α-tocopherol concentrations were presented on a total lipids basis, horses fed corn oil supplement had a higher plasma α-tocopherol to total lipids ratio compared with fish oil—fed horses at all time points. Similar to α-tocopherol concentrations, the α-tocopherol to total lipids ratio at week 0 was the lowest compared with all other time points. Because horses fed corn oil had a higher plasma α-tocopherol to total lipids ratio compared with fish oil—fed horses at week 0, initial values were also used as a covariate to test for differences between diets across time. Covariate analysis showed no dietary effect.

LTB_4 and LTB_5 Quantification

Neither LTB. LTB, production by stimulated peripheral blood neutrophils was significantly different between the 2 groups of horses at week 0 (Fig 1). Although LTB_4 and LTB_5 production within the group of horses fed corn oil increased slightly (nonsignificant) from baseline to week 12, the horses fed fish oil had a significant increase in production of both leukotrienes at 12 weeks. The ratio of LTB_5 to LTB_4 was also significantly higher at 12 weeks compared with baseline values within the group of horses fed fish oil.

The ratio of EPA to AA in the plasma across dietary treatments and time periods (weeks 0 and 12) was correlated (correlation coefficient = 0.90, $P < .0001$) to the ratio of LTB, to LTB, After feeding horses a corn oil supplement for 12 weeks, the EPA : AA ratio was not different from that of week 0. However, after feeding horses a fish oil supplement for 12 weeks, the ratios for EPA : AA and LTB_5 : LTB_4 were both significantly increased compared with those at week 0 (4.6-fold and 9.4-fold, respectively).

Serum Chemistries

For all serum analytes measured, only calcium and magnesium were influenced by dietary treatment (Table 4). Overall, mean serum calcium concentration was greater for horses fed fish oil compared with corn oil supplements, with mean calcium concentrations across diets being

highest at 12 weeks. Serum calcium concentrations in corn oil— fed horses declined from week 0 to week 8 and then increased at week 12. In contrast, fish oil—fed horses showed a tendency for a linear increase in serum calcium concentrations over time. Overall, the mean serum magnesium concentration was greater for corn oil—fed horses than for fish oil—fed horses. Mean serum magnesium concentrations across diets declined with time. Fish oil—fed horses exhibited a linear decline in serum magnesium concentrates,

Table 2. Effect of feeding horses diets supplemented with oils that differed in their (n-6) and (n-3) fatty acid content on plasma fatty acid profiles after 6, 8, and 12 weeks compared with baseline (week 0).[a] Oil supplementation was discontinued at 14 weeks and plasma fatty acid profiles were reassessed 4 weeks later (week 18).

Time	0 weeks			6 weeks			8 weeks			12 weeks			18 weeks			*P* Values[b]		
Oil Supplement (n-6) : (n-3)	Corn Oil 68.1 : 1	Fish Oil 0.12 : 1	Pooled SEM	Corn Oil 68.1 : 1	Fish Oil 0.12 : 1	Pooled SEM	Corn Oil 68.1 : 1	Fish Oil 0.12 : 1	Pooled SEM	Corn Oil 68.1 : 1	Fish Oil 0.12 : 1	Pooled SEM	Corn Oil 68.1 : 1	Fish Oil 0.12 : 1	Pooled SEM	Diet	Week	Diet × Week
Fatty Acid[c,d]																		
C14 : 0	2.33	1.24	0.92	0.99	0.76	0.26	2.28	0.80	0.89	0.37[f]	0.87	0.18	3.36	4.94[f]	0.83	NS[g]	<.0001	0.14
C16 : 0	15.16	15.41	0.46	**11.92**[f]	**14.91**	0.56	**11.29**[f]	**15.01**	0.70	13.75	16.66	1.85	14.08	14.94	0.63	0.04	0.0001	0.003
C18 : 0	15.55	16.02	0.45	16.06	15.43	0.54	16.02	14.80[f]	0.55	18.62[f]	17.48	1.38	16.56[f]	16.18	0.50	NS	0.01	0.17
C20 : 0	0.39	0.46	0.06	0.40	0.34[f]	0.03	0.35	0.27[f]	0.05	0.33	0.72	0.20	0.44	0.46	0.02	NS	0.0012	0.18
C22 : 0	0.37	0.44	0.10	**0.32**	**0.70**[f]	0.09	**0.28**	**0.73**[f]	0.11	**0.17**	**0.67**[f]	0.10	0.31	0.25	0.03	0.01	0.04	0.002
C24 : 0	0.19	0.18	0.04	0.44	0.46	0.17	0.31	0.49[f]	0.12	0.22	0.38[f]	0.09	**0.33**[f]	**0.26**[f]	0.02	NS	0.01	0.17
Sum SFA[e]	33.99	33.73	1.22	30.13[f]	32.60	0.60	30.52[f]	32.12	1.34	33.46	36.79	2.79	35.08	37.03[f]	1.33	0.18	0.0002	NS
C14 : 1	0.17	0.13	0.06	0.11	0.09	0.06	0.11	0.16	0.06	0.14	0.22[f]	0.06	0.15	0.26	0.06	NS	<.02	0.08
C16 : 1(n-7)	1.21	1.06	0.12	**0.60**[f]	**1.96**[f]	0.09	**0.48**[f]	**1.99**[f]	0.09	**0.44**[f]	**2.18**[f]	0.23	1.13	1.20	0.08	0.0002	0.2	<.0001
C18 : 1(n-9)	16.34	15.57	1.76	**8.14**[f]	**9.30**[f]	0.31	**7.50**[f]	**9.11**[f]	0.16	9.77[f]	10.61[f]	1.44	13.32	14.24	1.10	NS	0.0001	NS
C20 : 1(n-9)	**0.39**	**0.16**	0.05	**0.24**[f]	**0.33**[f]	0.03	0.33	0.40[f]	0.05	0.25	0.27	0.07	0.32	0.31[f]	0.05	NS	NS	0.01
C22 : 1(n-9)	0.49	0.19	0.24	0.00[f]	0.05	0.02	0.04[f]	0.11	0.05	0.34	0.22	0.19	0.22	0.18	0.10	NS	0.01	NS
C24 : 1	0.30	0.26	0.05	**0.39**	**0.65**[f]	0.04	**0.47**	**0.93**[f]	0.11	0.29	0.60[f]	0.13	0.19	0.24	0.05	0.01	<.0001	0.005
Sum MUFA[e]	18.90	17.36	1.76	**9.48**[f]	**12.38**[f]	0.32	**8.93**[f]	**12.70**[f]	0.32	11.23[f]	14.10	1.54	15.33[f]	16.44	1.14	0.1	<.0001	0.11
C18 : 2(n-6)	37.74	39.72	2.48	**49.04**[f]	**26.84**[f]	1.03	**48.97**[f]	**26.80**[f]	0.72	**47.47**[f]	**29.88**[f]	3.08	**39.85**	**34.14**[f]	1.55	<.0001	NS	<.0001
C18 : 3(n-3)	2.44	2.84	0.83	**0.85**[f]	**1.19**[f]	0.11	0.91[f]	0.96[f]	0.19	0.50[f]	0.73[f]	0.28	2.99	2.89	0.47	NS	<.0001	NS
C20 : 2(n-6)	0.30	0.32	0.07	**0.32**	**0.18**[f]	0.05	0.30	0.34	0.07	**0.49**[f]	**0.27**	0.06	0.09[f]	0.07[f]	0.03	NS	<.0001	0.01
C20 : 3(n-6)	0.04	0.26	0.16	**0.10**	**0.39**	0.07	**0.09**	**0.23**	0.05	0.03	0.15	0.09	0.09	0.07	0.04	0.05	0.08	0.1
C20 : 4(n-6)	0.75	0.76	0.29	**0.68**	**3.85**[f]	0.29	**0.98**	**3.68**[f]	0.29	**0.49**	**4.09**[f]	0.29	1.29	1.85[f]	0.29	<.0001	<.0001	<.0001
C20 : 5(n-3)	0.21	0.38	0.10	**0.38**	**9.06**[f]	0.34	**0.35**	**8.61**[f]	0.24	**0.31**	**8.48**[f]	0.78	0.24	0.20	0.07	<.0001	<.0001	<.0001
C22 : 5(n-3)	0.10	0.15	0.04	**0.16**	**0.86**[f]	0.05	**0.16**	**0.88**[f]	0.03	**0.05**	**0.52**[f]	0.15	0.15	0.23	0.10	0.001	<.0001	<.0001
C22 : 6(n-3)	0.11	0.11	0.02	**0.17**	**5.62**[f]	0.16[g]	**0.30**	**5.97**[f]	0.23	**0.15**	**5.11**[f]	0.26	0.24	0.34[f]	0.09	<.0001	<.0001	<.0001
Sum PUFA[e]	41.68	44.53	1.88	**51.70**[f]	**48.00**	0.82	**52.05**[f]	**47.47**	0.50	49.48	49.23	3.55	**44.93**	**39.78**[f]	1.23	0.13	<.0001	0.1
Sum (n-6)[e]	38.82	41.06	2.18	**50.14**[f]	**31.27**[f]	0.95	**50.33**[f]	**31.06**[f]	0.60	**48.48**[f]	**34.39**	3.69	**41.32**	**36.12**	1.59	0.0002	NS	<.0001
Sum (n-3)[e]	2.86	3.47	0.80	**1.57**	**16.73**[f]	0.50	**1.72**	**16.41**[f]	0.50	**1.00**	**14.84**[f]	1.13	3.61	3.66	0.43	<.0001	<.0001	<.0001
Ratio (n-6) : (n-3)	19.07	17.52	6.13	**34.94**[f]	**1.89**[f]	3.52	**32.31**	**1.91**[f]	3.69	**56.58**[f]	**2.45**	8.77	12.26	10.42	1.45	0.0003	0.003	<.0001
Ratio EPA : AA	**0.14**	**0.44**	0.11	**0.56**[f]	**2.38**[f]	0.16	**0.35**	**2.35**[f]	2.18	**0.24**	**2.04**[f]	0.16	0.19	0.11[f]	0.05	<.0001	<.0001	<.0001
Total FA %	94.58	95.63	2.00	91.31	92.98	1.11	91.50	92.29	1.56	94.18	100.12	3.12	95.35	93.25	1.39	NS	0.05	NS

[a] Bold text indicates mean values are significantly ($P < .05$) different between dietary treatments at that time point.

[b] *P* values for main effects of diet, week, and their interaction.

[c] Least squares means ± pooled SEM.

[d] Fatty acid data are expressed as grams/100 grams fatty acids.

[e] See Table 1 for an explanation of footnote.

[f] Significantly different ($P < .05$) from week 0 within dietary treatment.

[g] Nonsignificant, $P < .20$.

Table 3. Effect of feeding horses diets supplemented with oils that differed in their (n-6) and (n-3) fatty acid content on plasma lipid and α-tocopherol concentrations after 6, 8, and 12 weeks compared with baseline (week 0).[a]

Time	0 Weeks			6 Weeks			8 Weeks			12 Weeks			*P* Values[b]		
Oil Supplement	Corn Oil	Fish Oil	Pooled SEM	Corn Oil	Fish Oil	Pooled SEM	Corn Oil	Fish Oil	Pooled SEM	Corn Oil	Fish Oil	Pooled SEM	Diet	Week	Diet × Week
Cholesterol (mg/dL)	79.7	75.8	7.7	114.5[e]	107.9[e]	7.7	103.2[e]	94.4[e]	8.1	102.1[e]	94.0[e]	0.21	NS[c]	<.0001	NS
Triacylglycerol (mg/dL)	25.7	28.3	7.1	15.0	15.9[e]	2.7	19.5	19.5	2.7	16.8	18.6	3.5	NS	0.003	NS
Total Lipids (mg/dL)	105.4	104.2	9.9	129.8[e]	124.0[e]	9.9	123.4[e]	115.2	10.2	119.2	112.5	10.1	NS	<.006	NS
α-tocopherol (μg/mL)	4.3	2.8	0.8	**7.7**[e]	**5.0**[e]	0.8	7.5[e]	5.1[e]	0.9	7.0[e]	4.7[e]	0.8	0.08	<.0001	NS
α-tocopherol per total lipids[d] (μg/mg mass ratio)	**4.23**	**2.80**	0.45	**5.57**[e]	**3.90**[e]	0.45	**5.81**[e]	**4.44**[e]	0.47	**5.68**[e]	**4.17**[e]	0.47	0.03	<.0001	NS

[a] Bold text indicates mean values are significantly ($P < .05$) different between dietary treatments at that time point. All values are least squares means ± SEM.

[b] *P* values for main effects of diet, week, and their interaction.

[c] Nonsignificant at $P > .20$.

[d] Using initial values as covariates, dietary effect was not significant.

[e] Significantly different ($P < .05$) from week 0 within dietary treatment.

whereas corn oil—fed horses showed a decline from week 0 to week 8, and no difference between weeks 8 and 12. Other serum analytes measured were all within expected normal ranges for mature horses.

Discussion

All horses readily consumed their corn oil or fish oil supplements. As expected, plasma fatty acid profiles of horses reflected PUFA content of the oils fed. Total plasma (n-6) PUFA increased in horses fed corn oil, whereas total plasma (n-3) PUFA increased in horses fed fish oil with a concomitant decrease in plasma (n-6) PUFA. Although horses that consumed the corn oil supplement had an increase in linoleic acid, they did not have a corresponding increase in plasma AA. Paradoxically, horses that consumed the fish oil supplement had an increase in plasma AA, which appeared to reflect the presence of AA in the fish oil supplement. The increase in plasma AA in horses fed the fish oil supplement was inconsistent with results of other studies, using other animal species.[18-20]

Other researchers have reported similar results to ours when horses were fed an (n-6) fatty acid supplement.[21] Plasma phospholipid profiles in that study were determined after 10 and 16 months of dietary supplementation with (n-6) PUFA. The amount of linoleic acid was, interestingly, 36 to 45 times higher compared to that of AA in plasma phospholipids. In addition, analysis of cholesterol esters, triglycerides, and free fatty acid revealed no detectable AA levels. In our study, plasma linoleic acid concentrations were 50- to 95-fold higher than AA concentrations in horses fed the (n6) fatty acid supplement. These data suggest that AA is not readily synthesized from linoleic acid in horses.

In another study, horses were fed a flaxseed oil—supplemented pellet for 16 weeks, and plasma linoleic acid concentrations increased because flaxseed oil had a high concentration of linoleic acid in addition to α-linolenic acid.'" Similar tion of linoleic acid in addition to α-linolenic acid.[21] Similar to the results we report here, plasma AA levels in those flaxseed oil—supplemented horses were not increased in association with increased plasma linoleic concentrations.

Linoleic acid remained elevated in plasma of horses fed diets enriched in corn oil throughout the 12-week dietary supplementation period. One explanation for why horses that consumed the corn oil supplement had higher levels of plasma linoleic acid without an increase in plasma AA levels is that linoleic acid was not metabolized to AA by these horses. The enzymes involved in conversion of linoleic acid to AA include Δ6 desaturase, Δ5 desaturase, and an elongase. In our study, horses fed the corn oil supplement had very low plasma concentrations of fatty acids derived from enzymatic action of these enzymes. The high plasma linoleic acid concentration in this group of horses reflects the large amount of linoleic acid that was contained in the corn oil supplement. The increase in plasma AA concentrations noted in horses fed fish oil was more likely a result of exogenous AA contained in the fish oil. Activity of the Δ6deexogenous AA contained in the fish oil. Activity of the Δ6desaturase enzyme is affected by concentrations of (n-6) and (n-3) PUFA. Fatty acids that are substrates for this enzyme, 18 : 2(n-6) and 18: 3(n-3), can competitively inhibit metabolism of each other.[23] It is also possible that horses lack one or more of the enzymes involved in conversion of

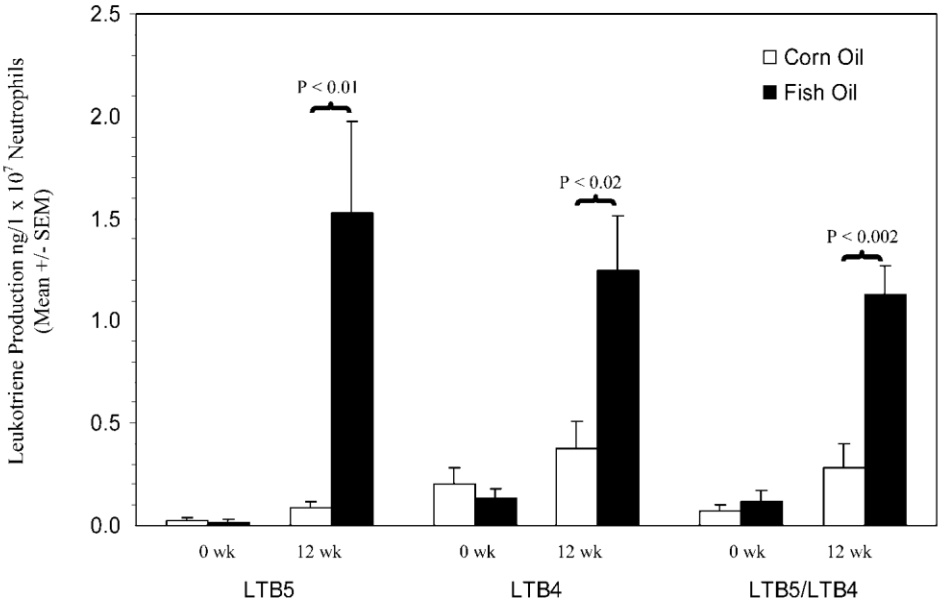

Fig 1. Effect of feeding horses diets for 12 weeks that differed in the (n-6) and (n-3) fatty acid content on the production of LTB_5 and LTB_4 by stimulated peripheral blood neutrophils. Data are expressed as nanograms per 1×10^7 cells (mean ± SEM). The ratio of LTB_5 to LTB_4 is also shown (mean ± SEM). Mean values differed significantly ($P < .05$) between dietary treatments at 12 weeks as indicated.

linoleic acid to AA. This phenomenon has previously been documented in wild and domestic cats [24-26] The Δ6 desaturase enzyme is the first enzyme in the metabolic pathway from linoleic acid to AA. It is also the rate-limiting enzyme; thus, it limits conversion of linoleic acid to AA even though there may be an abundance of linoleic acid in plasma.

Four weeks after the diets were discontinued, plasma fatty acid profiles were similar to those observed before dietary intervention. This study shows that plasma fatty acid profiles in horses are altered after 6 weeks of dietary fatty acid supplementation, and that changes return to predietary levels by 4 weeks after discontinuing fatty acid supplementation.

Horses in this study received a vitamin/mineral supplement that provided 800 IU of α-tocopherol/day to each ment that provided 800 IU of α-tocopherol/day to each horse. Plasma α-tocopherol measurements in our horses showed that plasma concentrations of α-tocopherol exceeded 2.0 μg/mL after 12 weeks of PUFA supplementation, which is considered the minimally adequate level for horses.[27] Some studies,[28] but not necessarily al1,[29] have shown that high intakes of (n-3) fatty acids lower plasma concentrations of α-tocopherol. In our study, dietary α-tocopherol supplementation of 800 IU/day resulted in increased plasma concentration of α-tocopherol in both groups of horses.

Plasma α-tocopherol concentrations were also expressed Plasma α-tocopherol concentrations were also expressed as a mass ratio between α-tocopherol and total lipid (cho-

Table 4. Effect of feeding horses diets supplemented with oils that differed in their (n-6) and (n-3) fatty acid content on selected serum biochemistries after 8 and 12 weeks compared with baseline (week 0).[a,b]

Time	0 Weeks			8 Weeks			12 Weeks			*P* Values[c]		
Oil Supplement	Corn Oil	Fish Oil	Pooled SEM	Corn Oil	Fish Oil	Pooled SEM	Corn Oil	Fish Oil	Pooled SEM	Diet	Week	Diet × Week
SUN[d] (mg/dL)	11.0^{f}	12.0^{f}	0.62	9.6^{f}	11.2^{f}	1.26	13.2^{g}	14.8^{g}	1.34	NS[e]	<0.0001	NS
Glucose (mg/dL)	97.0^{f}	97.6^{f}	2.11	93.0^{f,g}	92.5^{f,g}	2.35	88.8^{g}	86.8^{g}	2.35	NS	0.0023	NS
Total protein (g/dL)	6.28^{f,g}	6.14^{f,g}	0.11	6.24^{g}	6.00^{g}	0.13	6.58^{f}	6.48^{f}	0.13	0.13	0.011	NS
Albumin (g/dL)	2.94^{f}	3.00^{f}	0.10	3.06^{f,g}	3.18^{f,g}	0.11	3.14^{g}	3.25^{g}	0.11	NS	0.01	NS
Total bilirubin (mg/dL)	1.2^{g}	1.4^{g}	0.20	1.6^{f}	1.7^{f}	0.21	1.2^{g}	1.6^{f,g}	0.21	NS	0.001	NS
Sodium (mEq/L)	139.4^{f}	138.4^{f,g}	0.57	138.0^{g}	137.9^{g}	0.60	139.0^{f}	138.9^{f}	0.60	NS	0.0022	0.18
Potassium (mEq/L)	3.68	3.72^{g}	0.22	3.72	3.92^{g}	0.08	4.04	4.28^{f}	0.09	NS	0.003	NS
Calcium (mg/dL)	11.7^{f}	11.7^{g}	0.20	**11.2**^{g}	**12.0**^{f,g}	0.04	11.9^{f}	12.2^{f}	0.23	0.03	0.04	0.09
Magnesium (mg/dL)	1.8^{f}	1.7^{f}	0.04	1.7^{g}	1.7^{f}	0.02	1.7^{g}	1.6^{g}	0.02	0.03	0.004	0.07

[a] Bold text indicates mean values are significantly ($P < .05$) different between dietary treatments at that time point.
[b] All values are least squares means ± pooled SEM.
[c] *P* values for main effects of diet, week, and their interaction.
[d] Serum urea nitrogen.
[e] Not significant at $P > .20$.
[f,g] Different superscripts indicate mean values are significantly ($P < .05$) different within dietary treatment.

lesterol and triglyceride), because this provides the most accurate indicator of plasma α-tocopherol concentration.curate indicator of plasma α-tocopherol concentration.[16,30] The ratio of α-tocopherol to total lipids was increased in corn oil—supplemented horses compared with fish oil—supplemented horses before dietary oil supplementation began (week 0). Although this ratio increased with time in both groups of horses, it remained higher in the group of horses supplemented with corn oil at 6, 8, and 12 weeks. It is likely that this difference between groups represented group/animal effects present at the initial sampling time (week 0) that persisted. We have previously shown in dogs that plasma αtocopherol concentration is not dependent on dietary ratio of (n-6) and (n-3) fatty acids when α-tocopherol is expressed relative to the total lipid content of plasma.[30]

Supplementing the horse diet with either (n-6) or (n-3) fatty acids increased plasma cholesterol concentrations, which differs from human beings and dogs. [18-31] Plasma triglyceride concentrations were not different between the two groups of horses; however, both groups had decreased triglyceride concentrations after 12 weeks of supplementation compared with baseline values. Fish oil supplementation in humans is also associated with a reduction in triglyceride levels.[31] The results of the current study differ from the results of another study,[22] which showed no change in serum triglyceride concentrations after horses were fed flaxseed oil—supplemented pellets for 16 weeks.

The production of LTB_5 and LTB_4 from equine peripheral blood neutrophils reflects the plasma concentration of substrates EPA and AA, respectively, from which they are derived. Dietary supplementation of horses with fish oil resulted in high plasma concentrations of EPA and DHA and, unexpectedly, high plasma concentrations of AA. Consequently, those horses fed fish oil supplements had higher levels of the corresponding leukotrienes (LTB_5 and LTB_4). Dietary supplementation of horses with corn oil resulted in high plasma concentrations of linoleic acid, but not AA. It is apparent that horse neutrophils stimulated with calcium ionophore do not readily produce LTB4 in the absence of AA. Conversely, neutrophils from dogs fed 5 :1 and 10 :

1 (n-6) to (n-3) fatty acid ratios synthesized 30 to 33% less LTB4 and 370 to 500% greater LTB_5 after 6 and 12 weeks of feeding compared with week 0. This decrease in LTB_4 production by neutrophils from dogs fed foods with low (n6) to (n-3) fatty acid ratios is consistent with the decrease in plasma AA concentration in dogs fed fish oil supplements,18 and differs from the increase in plasma AA concentrations and increase in LTB_4 production by neutrophils from horses fed fish oil supplements reported here.

In another study, stimulated monocytes from horses fed a pelleted ration that contained 8% linseed oil for 8 weeks produced less LTB_4 than control horses.[32] Our fish oil—supplemented horses had increased plasma AA, which likely contributed to increased production of LTB4. The type of cells stimulated, differences in dietary fat concentrations and source, and different methodologies used to determine fatty acid content (plasma versus monocyte cell membranes) may account for the observed differences between these two studies.

It has been shown that exogenous AA is necessary for cultured equine neutrophils to produce leukotrienes when stimulated with calcium ionophore.[33] Results of our study suggest that a diet rich in (n-3) PUFA caused an increase in production of LTB_4 likely because AA from fish oil is incorporated into cell membrane phospholipids. However, a diet rich in (n-3) PUFA also increased production of LTB_5 which is less biologically active than LTB_4, and may have inhibitory effects on the function of LTB_4.[33] Thus, it is unclear to what extent increased production of LTB_4 in the horse may be harmful, based on concurrent increased production of LTB_5

In the current study, horses consuming fish oil supplements showed a strong correlation (correlation coefficient = 0.93) between the ratio of EPA to AA in plasma and the ratio of LTB_5 to LTB_4 synthesized by stimulated neutrophils. Similarly, a linear relationship has been demonstrated between the ratio of EPA to AA in cell membrane phospholipids and the ratio of LTB_5 to LTB_4 produced by rat peritoneal exudate cells in vitro.20 In horses consuming the fish oil supplement, the ratio of plasma EPA to AA and the ratio of LTB_5 to LTB_4 production both increased with time, and ratios were higher in horses receiving the fish oil supplement than in horses receiving the corn oil supplement. The strength of the correlation in horses fed fish oil compared with horses fed corn oil would suggest that dietary manipulation of fat has a direct effect on the LTB_5 to LTB_4 ratio. Corn oil supplements had no added AA or EPA, and the LTB_5 to LTB_4 ratio was dependent on previously incorporated membrane fatty acids. If the ratio of LTB, to LTB, is important in horses as it is in other species,20, 34-36 then an equine diet high in (n-3) PUFA is preferable to a diet high in (n-6) PUFA in terms of decreasing proinflammatory leukotriene synthesis.

Giving horses a supplement of fish oil for 12 weeks resulted in increased serum calcium and decreased serum magnesium concentrations, whereas supplementation with corn oil over the same period resulted in decreased serum magnesium. However, all values were within expected normal ranges for mature horses. For all other serum chemistries measured, no effects of corn or fish oil supplementation were found, which is consistent with the results of other studies.[22]

In summary, we have shown that supplementation with fish oil not only increased production of the proinflammatory eicosanoid LTB_4, but also increased production of the less inflammatory

eicosanoid LTB_5. The ratio of plasma EPA to AA corresponded with the ratio of LTB_5 to LTB_4 produced by stimulated equine neutrophils. If the ratio of LTB_5 to LTB_4 concentrations is important in determining how inflammatory processes are mediated, then investigations should be conducted to determine the optimum ratio of LTB_5 to LTB_4 to decrease inflammation.

Footnotes

a. Pyrantel pamoate, Strongid Paste, Pfizer, Inc., U.S. Animal Health Operations, New York, NY
b. Kindly provided by Omega Protein Inc., Reedville, VA
c. Mazola, CPC International, Englewood Cliffs, NJ
d. Horse Guard Inc., Redmond, OR
e. Dairy One Laboratory, Ithaca, NY
f. Sigma Chemical Co., St. Louis, MO
g. DMSO, Syntex, West Des Moines, IA
h. Cayman Chemical_ Co., Ann Arbor, Mt
i. Sep-Pak Classic, Waters Inc., Milford, MA
j. Nova-pak, 3.9 mm X 300 mm, 60A pore size, 4 p.m particle size, Waters Inc., Milford, MA
k. Nova-pak Sentry, 3.9 mm X 20 mm, Waters Inc., Milford, MA
l. Model HOB, Beckman, Fullerton, CA
m. System Gold Model 166, Beckman, Fullerton, CA
n. Perkin Elmer LC1-100 Laboratory Computing Integrator, Boston, MA
o. Bio-Tek Instruments Inc., Winooski, VT
p. Roche Inc., Somervdle, NJ

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